Identification and Subcellular Distribution of Cornified Envelope Precursor Proteins in the Transformed Human Keratinocyte Line SV-K14

Serge Michel, Ph.D., Rainer Schmidt, Ph.D., Shirley M. Robinson, B.Sc., Braham Shroot, Ph.D., and Uwe Reichert, Ph.D.

Centre International de Recherches Dermatologiques (CIRD), Sophia Antipolis, Valbonne, France

SV-40 transformed human foreskin keratinocytes (line SV-K14) develop under conditions of serum starvation the competence to form cornified envelopes that are characteristic of terminally differentiating epidermal cells. In this cell line, the final assembly of the envelope does not occur spontaneously but must be induced using a calcium ionophore.

Five potential precursor proteins with molecular weights of 140K, 90K, 61K, 53K, and 36K, respectively, could be detected in the extracts of envelope competent and noncompetent cells. The 61 kD and the 36 kD precursors were specifically decorated in immunoblots when using an antiserum directed against the purified cornified envelope of SV-K14 cells. The 140 kD protein was identified as involucrin by means of a commercial anti-involucrin antibody. Part of the 61 kD protein was found to be inserted into the plasma membrane after the cells gained envelope competence.

The set of precursor proteins used by SV-K14 cells differed markedly from those described in the literature for epidermal cells in vivo and for normal human keratinocytes in vitro. Furthermore, cyanogen bromide cleavage of purified envelopes from transformed and normal keratinocytes revealed a completely different peptide pattern. This indicates that the exact molecular composition of the cornified envelope may not be strictly determined and may vary according to the availability of potential substrate proteins at the very moment when the cross-linking enzyme, the plasma membrane associated transglutaminase, becomes functional. *J Invest Dermatol 88:301–305, 1987*

During terminal differentiation of epidermal keratinocytes a highly insoluble structure, the cornified envelope, is formed beneath the plasma membrane [1–3]. This envelope is synthesized by the enzyme transglutaminase (EC 2.3.2.13) which catalyzes, in the presence of calcium ions, the γ-glutamyl-ε-lysine isopeptide cross-linking [4–7] of precursor proteins [8–10].

Although the synthesis of this structure occurs spontaneously in normal keratinocytes, cells from the transformed line SV-K14 require (1) serum starvation to gain envelope competence and, additionally, (2) challenge with a calcium ionophore for the final assembly of the envelope [11,12]. Thus, SV-K14 cells provide an excellent means to study the mechanism of envelope formation since they can be obtained in 3 different physiologic states that can be considered to mimic different stages of terminal differentiation: (1) proliferative, nondifferentiated cells in the presence of serum, (2) differentiating, envelope-competent cells under conditions of serum starvation, and (3) differentiated, envelope-containing cells after calcium ionophore treatment.

Using this model in a recent publication [12], we reported that development of envelope competence is closely related to the appearance of transglutaminase activity in the plasma membrane. This enzyme is referred to by other authors as "particulate transglutaminase" [13,14]. In the present paper, we investigate the appearance of envelope precursors in the 3 different cellular stages defined above and their possible subcellular redistribution during the process of terminal differentiation. Furthermore, we compare the electrophoretic peptide pattern obtained after cyanogen bromide cleavage of the envelopes obtained from transformed and normal human keratinocytes in culture.

Manuscript received June 9, 1986; accepted for publication August 26, 1986.

Reprint requests to: Serge Michel, Ph.D., Centre International de Recherches Dermatologiques, Sophia Antipolis, F-06565 Valbonne Cedex, France.

Abbreviations:

BSA: bovine serum albumin
DMEM: Dulbecco's modification of Eagle's medium containing 5.5 mM glucose and 4 mM glutamine
DTE: dithioerythritol
EGTA: ethylene glycol-bis-(2-aminoethylether)-N,N′-tetraacetic acid
F12: Ham's medium F12
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline without calcium or magnesium
SDS: sodium dodecyl sulfate

MATERIALS AND METHODS

Chemicals Culture media and fetal calf serum were obtained from Flow Laboratories and GIBCO. Aprotinin, calcium ionophore A23187, and deoxyribonuclease were purchased from Boehringer (Mannheim). Polyacrylic acid, *M*, 90,000, was from Aldrich Chemicals. Rabbit anti-involucrin antibody was obtained from Biomedical Technologies. A 30% (w/v) stock solution of cationic silica microbeads was kindly provided by Dr. B. Jacobson (Department of Biochemistry, University of Massachusetts, Amherst). Products for gel electrophoresis were from Bio-Rad, Sigma, and Serva. Donkey antirabbit IgG, peroxidase-linked $F(ab')_2$ fragment, [^{35}S]methionine, and EN^{3}HANCE were obtained from

0022-202X/87/$03.50

New England Nuclear. Nitrocellulose paper was from Schleicher and Schuell.

Cells and Culture Conditions The SV-40 transformed human foreskin keratinocyte line SV-K14 (a kind gift from Dr. B. Lane, Imperial Cancer Research Fund, London) was used after about 20 passages. The cells were grown at 37°C (5% CO_2, humidified atmosphere) in Dulbecco's Modification of Eagle's medium containing 5.5 mM glucose and 4 mM glutamine/Ham's medium F12 (DMEM/F12) (1:1) containing 100,000 U penicillin, 100 mg streptomycin, and 250 μg amphotericin B per liter of medium, which was supplemented with 5–10% (v/v) fetal calf serum depending on the batch. Cornified envelope competence was achieved by culturing confluent cells for 4 days in serum-free medium, with a medium change after 2 days.

Trypsination of the Cells Cells were washed twice with phosphate-buffered saline (PBS) before being treated for 8 min at 37°C with PBS containing 0.04% (w/v) trypsin and 1% (w/v) EDTA. The trypsination was stopped by the addition of fetal calf serum to a final concentration of 20% (v/v).

Assay of Cornified Envelope Competence The procedure of Cline and Rice [15] was used with some modifications. Cells (4×10^5) were incubated in 0.5 ml DMEM/F12 (1:1) containing 10 μg of the calcium ionophore A23187. After 3 h, 50 μl of 20% (w/v) sodium dodecyl sulfate (SDS) containing 1 mg dithioerythritol (DTE) was added, before heating the suspension for 5 min at 100°C. After cooling, 10 μl of a DNAase solution (1 mg/ml) was added to prevent aggregation of the envelopes, which were then counted with a hematocytometer.

Cell Fractionation Extracts of total proteins were prepared by suspending cells in electrophoretic sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol, followed by a brief sonication (SONICATOR, model W-10), heating for 2 min at 100°C, and subsequent centrifugation. The protein content of the supernatant was determined by the method of Lowry, using bovine serum albumin as the standard.

Plasma membranes were purified by the microbead density perturbation technique [16] essentially as described [17]. Microbead-coated cells were lysed in a hypotonic buffer (5 mM Tris-HCl, pH 7.5, 1 mM EGTA (ethylene glycol-bis-(2-aminoethylether)N,N′-tetraacetic acid), 1 mM DTE, 1 μg aprotinin/ml). The suspension was homogenized in a tightly fitting Dounce glass homogenizer until the nuclei were broken (about 20 strokes). The plasma membranes were spun down at 300 *g* for 5 min and washed twice with lysis buffer. The supernatant was centrifuged for 30 min at 50,000 *g*. The resulting pellet was considered to be the particulate fraction, and the supernatant to be the cytosoluble fraction. The protein content of the different fractions was determined using the Bio-Rad protein assay.

Purification of Cornified Envelopes and Immunization Envelope competent cells (3×10^8) were treated with the calcium ionophore A23187 as described above. The envelopes were recovered by centrifugation and washed 4 times with serum-free DMEM/F12 (1:1) containing 2% (w/v) SDS and 0.2% (w/v) DTE; then washed twice with PBS. An aliquot of the preparation was subjected to SDS electrophoresis, which proved the absence of soluble proteins. Albino New Zealand rabbits, 2.5 kg, were injected intradermally at 15–20 sites on their flanks with 500 μg of the cornified envelopes, which had been suspended in 1 ml of sterile PBS and then emulsified with 1 ml of Freund's complete adjuvant. Three weeks after the primary injection, the rabbits were boosted subcutaneously and subscapularly with a further 500 μg of purified envelopes. Animals were bled from the ear vein 2 weeks after the final boost. Response was determined by immunoblotting.

Cyanogen Bromide Cleavage of Cornified Envelopes [18] The protein content of the envelopes was determined in an aliquot of the preparation by the Lowry procedure after digestion with proteinase K (50 μg/ml). The remaining envelopes were suspended in 70% formic acid, and 100 mg CNBr was added per mg protein. After 24 h at room temperature the reaction was complete and the mixture was diluted 1:5 with distilled water. After lyophilization, the peptides were suspended in Laemmli sample buffer [19] and separated on a 15% polyacrylamide gel.

Electrophoresis and Immunoblotting Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out under reducing conditions as described [19]. Six percent, 10%, and 15% gels were used and 60 μg of protein was routinely applied on each slot. The gels were stained with 0.2% (w/v) Coomassie Blue R 250 and destained in 50% (v/v) methanol containing 7.5% (v/v) acetic acid.

After metabolic labeling of cellular proteins with [^{35}S]-methionine, fluorography was performed by treating the gels with EN^{3}HANCE. The dried gels were exposed to Kodak DEF films at −70°C.

For immunoblotting experiments, the proteins were transferred to nitrocellulose paper as described [20]. The sheets were saturated for 12 h at room temperature in 10 mM Tris-HCl, pH 7.4, containing 140 mM NaCl and 3% (w/v) bovine serum albumin (BSA). They were then incubated with the diluted antiserum in the same buffer. The sheets were washed and incubated with a 1:100 dilution of donkey antirabbit IgG peroxidase-linked F(ab′)$_2$ fragment. Then they were stained by using the 4-chloro-1-naphthol hydrogen peroxide method [21].

RESULTS AND DISCUSSION

The experimental approach of Simon and Green [22] was used to identify proteins that are cross-linked during envelope formation. Serum-starved, i.e., envelope-competent cells, were subdivided into 3 groups (A–C). Group A received no further treatment. The remainder were challenged with the calcium ionophore, either in the absence (group B) or in the presence (group C) of 5 mM cystamine, a potent inhibitor of the cross-linking enzyme transglutaminase [23]. Only cells of group B synthesized cornified envelopes (Fig 1*A*) with a yield of almost 100%. In groups A and C, envelopes were essentially absent (less than 2%). Total protein extracts of these 3 cellular fractions were subjected to one-dimensional SDS-polyacryamide electrophoresis by using 6% and 10% gels. After Coomassie Blue staining, the electrophoretic patterns (Fig 2*a,b*) demonstrate a significant diminution of 4 soluble proteins in the extract obtained from cells after envelope formation (group B, lane C). This can be explained either by insolubilization of these proteins due to their insertion into the cornified envelope or by calcium-activated proteolytic digestion. The latter possibility is unlikely since, in the presence of cystamine, when transglutaminase was inhibited, diminution was much less pronounced (fraction C, lane D). Thus, these 4 proteins are potential candidates for envelope precursors. From the average of 8 independent experiments, their molecular weights were estimated to be 90K, 61K, 53K, and 36K, respectively, by comparison with commercially available standards. The protein band in the 200 kD region (lane B), which became substantial after

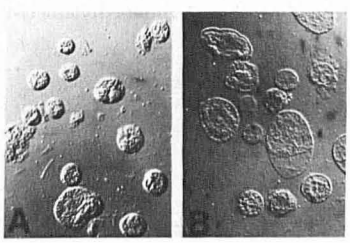

Figure 1. Nomarski contrast microphotographs of cornified envelopes obtained from the transformed line SV-K14 (*A*) and from normal human keratinocytes in culture (*B*).

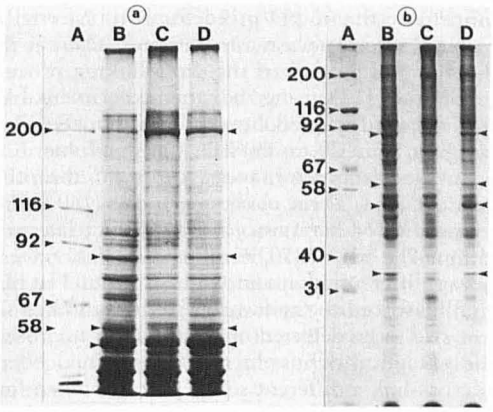

Figure 2. Electrophoretic analysis of solubilized SV-K14 proteins from envelope-competent cells without (*lane B*) and after challenge with 10 mg/liter ionophore A23187, in the absence (*lane C*) and in the presence (*lane D*) of 5 mM cystamine. Figure *a* represents a 6% and figure *b* a 10% gel. *Lane A* indicates, from top to bottom, the position of following molecular weight standards: myosine, β-galactosidase, phosphorylase b, bovine serum albumin, catalase, aldolase, DNAase I.

cells were permitted to synthesize an envelope, most probably represents still soluble products of the cross-linking process. This band is less pronounced in the presence of cystamine.

The proteins were electrophoretically transferred from the 10% gels to nitrocellulose paper and incubated with diluted antiserum obtained from rabbits after immunization with purified envelopes from SV-K14 cells. The antiserum led to the selective staining of the 61 kD and 36 kD proteins, whereas the 90 kD and 53 kD proteins did not react (Fig 3, lane B). Essentially no reaction with the antiserum was observed after envelope formation (Fig 4, lane B to be compared with lanes A and C). The preimmune serum served as the control, and decorated none of the described precursors (Fig 3, lane A). The treatment of the cells with calcium ionophore led, in the presence of cystamine, to an obvious reversion of the intensity of the 61 kD and 36 kD bands (Fig 4, lane C to be compared with lane A). One ad hoc explanation for this observation could be that the 61 kD protein is a cross-linked dimer of the 36 kD precursor and that the dimerization is not completely abolished in the presence of cystamine.

Involucrin, a 140 kD protein, is one of the major and specific

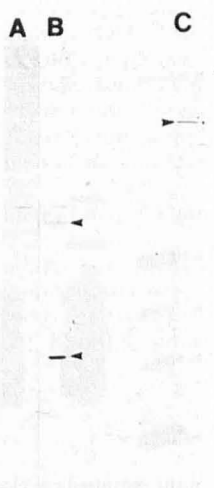

Figure 3. Immunoblot of solubilized SV-K14 proteins using rabbit anti-SV-K14-envelope antiserum (*lane B*), the corresponding preimmune serum (*lane A*), and a commercial anti-involucrin antibody (*lane C*).

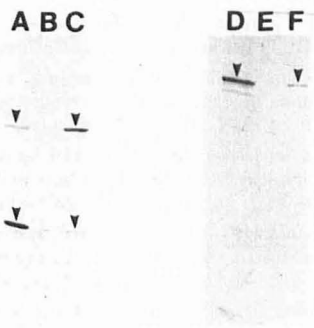

Figure 4. Immunoblot of solubilized SV-K14 proteins from envelope-competent cells without (*lanes A, D*) and after challenge with 10 mg/liter ionophore A 23187, in the absence (*lanes B, E*) and in the presence (*lanes C, F*) of 5 mM cystamine. The rabbit anti-SV-K14-envelope antiserum (*lanes A–C*) and the commercial anti-involucrin antibody (*lanes D–F*) were used to decorate the proteins.

envelope precursors in cultured normal human keratinocytes [22,24]. It has been reported, however, to disappear after transformation of these cells by Simian Virus-40 [25–27]. We agree with these results as far as Coomassie Blue-stained electropherograms are concerned (Fig 2), but, as seen from Fig 3, lane C, when a commercial anti-involucrin antibody was used in the immunoblot, a protein in the 140 kD position was decorated, and only in extracts obtained from competent cells that were not allowed to accomplish envelope formation (Fig 4, lanes D and F, to be compared with lane E). Thus, involucrin is present in sufficient quantity to be visualized by immunoblotting. The amount synthesized, however, is too low for detection by Coomassie Blue staining or classical immunofluorescence [25–27].

The question arose as to whether these 5 potential precursor proteins are newly synthesized during serum starvation or whether they already preexist in noncompetent cells. As shown in Figs 5 and 6, no differences between the protein patterns from competent and noncompetent cells could be detected, neither by Coomassie Blue staining (Fig 5, lanes A and B), nor by fluorography after metabolic protein labeling with [^{35}S]methionine (Fig 5, lanes C

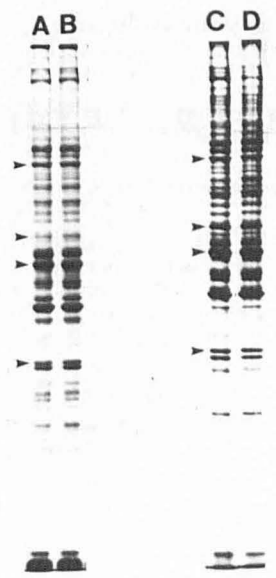

Figure 5. Electrophoretic pattern of solubilized SV-K14 proteins from envelope-competent (*lanes B, D*) and noncompetent cells (*lanes A, C*) obtained with Coomassie Blue staining (*lanes A, B*) and with fluorography after metabolic labeling of cellular proteins with [^{35}S]-methionine (*lanes C, D*). The *arrows* indicate the positions of precursor proteins.

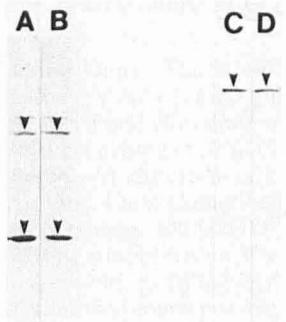

Figure 6. Immunoblot of solubilized SV-K14 proteins from envelope-competent (*lanes B, D*) and noncompetent cells (*lanes A, C*) using anti-SV-K14-envelope antiserum (*lanes A, B*) and anti-involucrin antibody (*lanes C, D*).

and D), nor by immunoblotting (Fig 6). This result indicates the existence in this cell line of proteins that are capable of being used for envelope formation, regardless of the physiologic state of the cells.

Our earlier finding that the development of envelope competence is accompanied by the appearance of transglutaminase activity in the plasma membrane prompted us to look into the subcellular distribution of the precursors in envelope-competent and envelope-noncompetent cells.

Three different subcellular fractions were prepared by means of the microbead density perturbation technique as described in *Materials and Methods:* (1) plasma membranes, (2) a particulate fraction consisting mainly of mitochondria and lysosomes, and (3) the "cytosoluble" fraction containing cytosol and light particulate material. Nuclei were largely destroyed during membrane preparation and contributed presumably to a large extent to the cytosoluble fraction.

The proteins were solubilized, electrophoretically separated, and blotted onto nitrocellulose before being treated with anti-involucrin and antienvelope antisera (Fig 7). There was almost no reaction with proteins from the particulate fraction. Involucrin and the 36 kD precursor were detected essentially in the cytosoluble fraction. The 61 kD protein was similarly distributed with the exception that it was also found in the plasma membrane of competent cells. This indicates that during development of envelope competence the 61 kD precursor is transferred from the cytosoluble pool into the plasma membrane. There it may serve as an anchorage protein to start the cross-linking process.

Simon and Green [22], using the same experimental approach, identified in cultured normal human keratinocytes 7 precursor proteins ranging from 70 to 330 kD. Only involucrin (140 kD) was found in the "submembranous cytosol," the others were membrane associated. Three of these proteins (140, 195, and 210 kD) were specific for keratinocytes, appearing late in terminal differentiation. The other 4 (70, 95, 100, and 330 kD) were present at all stages of differentiation and were also found in fibroblasts, which could be forced to form insoluble cross-linked fragments of sufficient size to be detected under the light microscope.

With the exception of involucrin, transformed keratinocytes obviously cross-link a different set of proteins when forming an envelope, which does, however, resemble the structure synthesized by normal keratinocytes (Fig 1*A* to be compared with Fig 1*B*).

Kubilus and Baden [28] identified in soluble extracts of cultured human keratinocyte and human epidermis 2 major transglutaminase substrates—A (125 kD) and B (12 kD)—by their reaction with dansylcadaverine. This technique has the limitation that it detects only γ-glutamyl-donating proteins whereas the procedure of Simon and Green is able to reveal both, ε-lysyl and γ-glutamyl donors. Substrate A, probably identical to involucrin and specific for differentiating cells, was expressed much more strongly in cultured cells than in epidermal extracts whereas substrate B, characteristic of malpighian cells, was detected equally in the extracts from both sources. With the same technique 2 further potential envelope precursors with M_r of 40K and 20K could be identified, but in minor quantities [29].

Zettergren et al [30] reported keratolinin, a 36 kD protein, to be the major in vivo constituent of human and bovine cornified envelopes [25]. This precursor consists of noncovalent 6 kD subunits that dissociate readily in the presence of detergents and thus is most likely not identical to our 36 kD protein. Furthermore, keratolinin has not yet been detected in vitro.

Comparing our findings with those described by the other authors mentioned above, we conclude that the formation of a cornified envelope during terminal differentiation requires (1) the existence of a functional transglutaminase in the plasma mem-

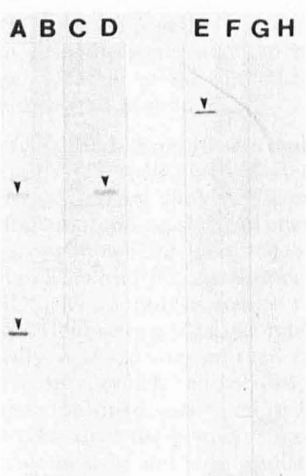

Figure 7. Immunoblot of precursor proteins in the cytosoluble fraction (*lanes A, E*), particulate fraction (*lanes B, F*), and plasma membrane fraction (*lanes C, D, G, H*) from envelope-competent (*lanes D, H*) and noncompetent (*lanes A–C, E–G*) cells using the anti-SV-K14-envelope antiserum (*lanes A–D*) and anti-involucrin antibody (*lanes E–H*).

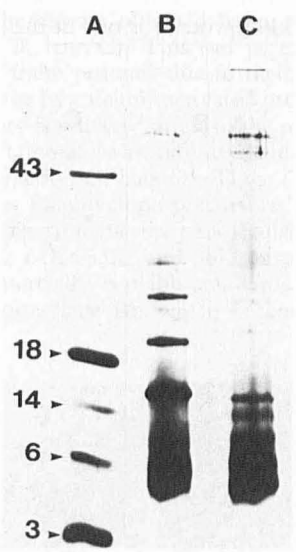

Figure 8. Peptide maps of the cornified envelopes from cultured normal keratinocytes (*lane B*) and SV-K14 cells (*lane C*) obtained after CNBr cleavage, SDS-PAGE on a 15% gel, and silver staining [32]. *Lane A* indicates from top to bottom the position of the following marker proteins: ovalbumin, β-lactoglobulin, lysozyme, bovine trypsin inhibitor, and insulin. Thirty micrograms of protein was applied on each slot.

brane [12] and (2) the availability of potential substrates for this enzyme. It is probable that at least some of the precursors have to be, or to become, membrane associated before cross-linking occurs. The set of precursors, which may be used by epidermal cells under different conditions, however, appears not strictly defined and may be recruited from preexisting proteins that can be used as substrates by the cross-linking enzyme.

The completely different peptide maps (Fig 8) obtained after CNBr cleavage of purified envelopes from SV-K14 cells and normal human keratinocytes cultured according to the technique of Rheinwald and Green [32] supports this view. Furthermore, CNBr peptide maps of the cornified envelopes from several healthy and psoriatic volunteers revealed striking differences between normal and lesional epidermis, but also interindividual variations (manuscript in preparation).

Taking all this information together, we hypothesize that during the late stages of terminal keratinocyte differentiation, when most of the intracellular organelles become destroyed, part of the waste proteins may be reused as building blocks for the formation of a cornified envelope. We call this working concept the "dustbin hypothesis."

We are indebted to Professor Hans Schaefer for his continuous interest and support, to Dr. Bruno A. Bernard for suggestions, to Dr. Gerard Pautrat for the Nomarski microphotographs, and to Catherine Dalbiez and Michelle Bronfort-Grangeret for their skillful technical assistance.

REFERENCES

1. Matoltsy AG, Balsano CA: A study of the components of the cornified epithelium of human skin. J Biophys Biochem Cytol 1: 339–360, 1955
2. Farbman AI: Plasma membrane changes during keratinization. Anat Rec 156:269–281, 1966
3. Hashimoto K: Cellular envelopes of keratinized cells of the human epidermis. Arch Klin Exp Dermatol 253:374–385, 1969
4. Sugawara K: Intermolecular cross-links in epidermal differentiation, in Biochemistry of Cutaneous Differentiation. Edited by M Seiji, IA Bernstein. Baltimore, University Park Press, 1977, pp 337–397
5. Rice RH, Green H: The cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked proteins. Cell 11:417–422, 1977
6. Rice RH, Green H: Relation of protein synthesis and transglutaminase activity to formation of cross-linked envelopes during terminal differentiation of cultured human epidermal keratinocytes. J Cell Biol 76:705–711, 1978
7. Rothnagel JA, Rogers GE: Transglutaminase-mediated cross-linking in mammalian epidermis. Mol Cell Biochem 58:113–119, 1984
8. Matoltsy AG: The membrane of horny cells, in Biochemistry of Cutaneous Differentiation. Edited by M Seiji, IA Bernstein. Baltimore, University Park Press, 1976, pp 93–109
9. Sun TT, Green H: Differentiation of the epidermal keratinocyte in cell culture: formation of a cornified envelope. Cell 9:511–521, 1967
10. Green H: Terminal differentiation of cultured human epidermal cells. Cell 11:405–415, 1977
11. Taylor-Papadimitriou J, Purkis P, Lane EB, McKay IA, Chang SE: Effect of SV40 transformation on the cytoskeleton and behavioural properties of human keratinocytes. Cell Differ 11:169–180, 1982
12. Schmidt R, Reichert U, Michel S, Shroot B, Bouclier M: Plasma membrane transglutaminase and cornified envelope competence in cultured human keratinocytes. FEBS Lett 186:201–204, 1985
13. Lichti U, Ben T, Yuspa S: Retinoic acid-induced transglutaminase in mouse epidermal cells is distinct from epidermal transglutaminase. J Biol Chem 260:1422–1426, 1985
14. Thacher SM, Rice RH: Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. Cell 40:685–695, 1985
15. Cline PR, Rice RH: Modulation of involucrin and envelope competence in human keratinocytes by hydrocortisone, retinyl acetate and growth arrest. Cancer Res 43:3203–3207, 1983
16. Chaney LK, Jacobson BS: Coating cells with colloidal silica for high yield isolation of plasma membrane sheets used for bilayer protein mapping. J Biol Chem 285:10062–10072, 1983
17. Schmidt R, Pautrat G, Michel S, Cavey MT, Gazith J, Dalbiez C, Reichert U: High yield purification of plasma membranes from transformed human keratinocytes in culture. J Invest Dermatol 85:50–54, 1984
18. Gross E: The cyanogen bromide reaction. Methods Enzymol 11:238–255, 1967
19. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1967
20. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354, 1979
21. Hawkes R, Niday J, Gordon J: A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 119:142–147, 1982
22. Simon M, Green H: Participation of membrane-associated proteins in the formation of cross-linked envelope of the keratinocyte. Cell 36:827–834, 1984
23. Lorand L, Siefring GE, Lowe-Krentz L: Formation of glutamyl lysine bridges between membrane proteins by a Ca^{2+}-regulated enzyme in intact erythrocytes. J Supramol Struct 9:427–440, 1978
24. Rice RH, Green H: Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. Cell 18:681–694, 1979
25. Hronis TS, Steinberg ML, Defendi V, Sun T-T: Simple epithelial nature of some simian virus-40 transformed human epidermal keratinocytes. Cancer Res 44:5797–5804, 1984
26. Okada N, Steinberg ML, Defendi V: Re-expression of differentiated properties in SV 40-infected human epidermal keratinocytes induced by 5-azacytidine. Exp Cell Res 153:198–207, 1984
27. Bernard BA, Robinson SM, Semat A, Darmon M: Reexpression of fetal characters in simian virus 40-transformed human keratinocytes. Cancer Res 45:1707–1716, 1985
28. Kubilus J, Baden HP: Isolation of two immunologically related transglutaminase substrates from cultured human keratinocytes. In Vitro 18:447–455, 1982
29. Kubilus J, Baden HP: Isopeptide formation in epidermis. Mol Cell Biochem 58:129–137, 1984
30. Zettergren JG, Peterson LL, Wuepper KD: Keratolinin: the soluble substrate of epidermal transglutaminase from human and bovine tissue. Proc Natl Acad Sci USA 81:238–242, 1984
31. Morrissey JH: Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307–310, 1981
32. Rheinwald JG, Green H: Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell 6:317–330, 1975